Assembly of a transmembrane \( \text{b-Type cytochrome} \) is mainly driven by transmembrane helix interactions

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Abstract

Folding, assembly and stability of \( \alpha \)-helical membrane proteins is still not very well understood. Several of these membrane proteins contain cofactors, which are essential for their function and which can be involved in protein assembly and/or stabilization. The effect of heme binding on the assembly and stability of the transmembrane \( \text{b-type cytochrome} \) \( \text{b}^{59} \) was studied by fluorescence resonance energy transfer. Cytochrome \( \text{b}^{59} \) consists of two monomers of a 44 amino acid long polypeptide, which contains one transmembrane domain. The synthesis of two variants of the \( \text{b}^{59} \) monomer, each carrying a specific fluorescent dye, allowed monitoring helix–helix interactions in micelles by resonance energy transfer. The measurements demonstrate that the transmembrane peptides dimerize in detergent in the absence and presence of the heme cofactor. Cofactor binding only marginally enhances dimerization and, apparently, the redox state of the heme group has no effect on dimerization.

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1. Introduction

Folding and stability of membrane proteins are still not well understood and many open questions remain to be answered. While \textit{in vivo} large transmembrane channels are involved in integration of single \( \alpha \)-helices or entire membrane proteins into a membrane (for recent reviews see \cite{1,2}), and the involvement of lipids or transmembrane chaperones, like YidC, in membrane protein folding is discussed \cite{3–5}, \textit{in vitro} studies with several membrane proteins have suggested that folding of \( \alpha \)-helical membrane proteins can be described by a simple two-stage model \cite{6,7}. In this model individual transmembrane helices integrate separately into a membrane (stage I) and afterwards the individual helices interact to form a higher ordered oligomer (stage II). This simplified view allows discrimination between energetic contributions from helix integration into a membrane and contributions of helix–helix interactions, and thus facilitates detailed studies of transmembrane helix–helix interaction.

Using a few basic model proteins, several studies have characterized the importance of single amino acids for the overall stability of a helix oligomer in micelles as well as in biological membranes.

As a paradigm for the second stage of the two-stage model, dimerization of the human glycophorin \( \text{A} \) transmembrane region has been studied extensively using different genetic, biochemical and biophysical techniques \cite{8–16}. The influence of cofactor binding on membrane protein folding and stability was measured using bacteriorhodopsin and retinal as a model system \cite{17–20}, followed by studies on a few other model proteins. In addition to bacteriorhodopsin, mainly the plant light harvesting complex II and the light harvesting complexes from purple bacteria have been studied \cite{21–31}. For bacteriorhodopsin it has been shown that the protein forms a folding intermediate without the retinal cofactor and adopts its final structure only upon cofactor binding \cite{17,18,32}. Since the cofactor is buried within the core of the...
seven-helix bundle, the protein most likely keeps an open con-
formation to allow incorporation and binding of retinal. In the case of the plant light harvesting complex II formation of stable secondary and tertiary structures is coupled with cofactor binding [28,29,33].

Several transmembrane proteins involved in charge transfer across membranes are b-type cytochromes and this class of proteins binds one or more heme cofactors non-covalently [34]. Transmembrane b-type cytochromes can be found in larger trans-
membrane protein complexes such as the succinate dehydroge-

nase (complex II), cytochrome bc1, and bc2 complexes, cytochrome bd and bo3 complexes, as well as in photosystem II of higher plants and cyanobacteria [35].

Cytochrome b559 is part of photosystem II and in vivo the protein is a heterodimer between one α- (PsbE) and one β-subunit (PsbF), which together bind one heme group non-covalently [36]. All residues critical for dimerization of the peptides as well as for heme binding are conserved in each individual subunit and, indeed, a homodimeric cytochrome b559-like protein (cytochrome heme binding are conserved in each individual subunit and, additional a PsbF-MalE fusion protein can be used to reconstitute a cytochrome b559-like protein in buffer containing only the non-

ionic detergent DDM [38].

The homodimeric cytochrome b559 represents the simplest transmembrane b-type cytochrome analyzed so far and can, there-

fore, serve as minimal model system to study the influence of heme binding on folding and stability of transmembrane b-type cyto-

chromes. In the present study chemically synthesized PsbF pep-
tides in combination with fluorescence resonance energy transfer was used to analyzed the effect of heme incorporation and of the heme redox state on transmembrane helix–helix interactions.

2. Materials and methods

2.1. Peptide synthesis

The PsbF transmembrane peptide (sequence in single-letter code: MATQ-

NPVPQTPFIFYWVLAVHTLAVPSYFGGAIAMQFIRCL) was synthe-

sized on an S-DVB (styrene-divinylbenzene) resin carrying an OCH2-PAM linker and using an in-situ neutralization protocol and HBTU activation for machine-

assisted Boc (tert-butyloxycarbonyl) chemistry [28]. All amino acids were obtained from Novabiochem. Solvents were purchased from Biosolve.

The peptide was deprotected and simultaneously cleaved from the resin using anhydrous HF. Subsequently it was precipitated with ether, and the crude product was dissolved in trifluoroethanol and subsequently diluted with three parts water containing 0.1% trifluoroacetic acid (TFA), and lyophilized. The peptide segment was purified by RP-HPLC on a C4-column from Vydac using a buffer consisting of 8 M urea, 200 mM NaPi and 17 mg/ml dodecylphosphocholine (DPC) at pH 8.0 mixed with 20% trifluoroethanol. 1.2 equivalents of each dye have been dissolved in DMSO and used in labeling reactions. Fluorescently labeled PsbF was purified by HPLC using the conditions described above. The molecular weight of PsbF-BODIPY was found to be 5724 Da (calculated MW 5724 Da).

2.2. Fluorescence spectroscopy

All fluorescence spectra were measured on an Aminco Bowman Series II fluorimeter with a slid width of 4 nm for the excitation and emission mono-

chromators. Samples were kept anaerobic with argon. Excitation and emission wavelengths are indicated in the figure legends.

2.3. Expression, purification of the PsbF-MalE fusion protein and analysis of dimerization

The psbF gene was amplified by PCR using genomic Synechocystis PCC 6803 DNA as a template and the gene was ligated to the expression vector pMal-

c2X (New England Biolabs) after restriction digestion of the PCR fragment and the vector with BamHI and HindIII. The sequence of the cloned DNA fragment was confirmed by DNA sequencing. Overexpression and purification of the PsbF-MalE fusion protein was done as described in detail recently [38].

For reconstitution in the presence of heme, the PsbF fusion protein was mixed with heme in a 2:1 ratio since cytochrome b559 is a homodimer, which binds one heme molecule. If indicated, SDS was added from a 0.5 M stock solution. For reduction 10 mM sodium dithionite was added to the solution from a 1 M stock solution. Samples were kept anaerobic with argon. Incorporation of heme and assembly of the cytochrome was tested by UV/Vis spectroscopy on a Perkin Elmer Lambda 35 instrument. Spectra were taken from 350 nm to 800 nm with a resolution of 0.5 nm. Commercially available hemine chloride (Sigma, Taufkirchen, Germany) was dissolved in 50% ethanol and the heme was solubilized by addition of NaOH. This heme solution was diluted fourfold into 50 mM Tris pH 8.0, 50 mM NaCl, 5 mM DDM buffer. The heme concentration was determined spectroscopically using an extinction coefficient of ε385 nm=56 M−1 cm−1.

To assess the influence of heme binding and the heme redox state on the thermodynamic stability of the PsbF dimer, about 5 mM PsbF-MalE fusion protein in 50 mM phosphate buffer pH 7.5, 50 mM NaCl, 5 mM DDM was mixed with 3.5 mM SDS. Samples were incubated for 2 h at room temperature and glutaraldehyde was added to a final concentration of 25 mM. Samples were then incubated in the absence of heme, or in the presence of a two-fold excess of heme. After addition of heme, the individual samples were kept free of oxygen by a stream of argon, which was directed over the solutions. For oxidation, 2 mM ferricyanide, for reduction 2 mM dithionite were added. Samples were incubated for about 16 h and analyzed on SDS gels containing 6 M urea.

2.4. GALLEX-assay

In vivo measurements of the homodimerization of the PsbF TM domain were done using the GALLEX system [8]. The psbF gene was amplified by PCR and ligated to the NheI/BamHI restriction digested plasmid pLM [8]. The correct insertion of the gene was checked by DNA sequencing. Ligation of a gene to this plasmid results in generation of an open reading frame, which encodes for a chimeric protein with a N-terminal fusion to the LexA DNA binding domain and a C-terminal fusion to the MalE domain. The resulting plasmid was transformed into E. coli SU101 and the GALLEX measurement was done according to the protocol published recently [8]. To introduce single point mutations into the psbF gene the Quickchange mutagenesis kit was used according to the manufacturer’s instructions (Stratagene).

3. Results

3.1. Dimerization of the PsbF protein studied with fusion proteins

It has recently been shown that cytochrome b559 assembles in vitro as well as in vivo [38]. In order to study the dimerization of...
the PsbF peptide in vivo using a genetic approach (GALLEX assay) [8], the psbF gene was expressed as a fusion to the DNA binding domain of the E. coli LexA protein. Heterologously expressed PsbF integrates into the E. coli cytoplasmic membrane and binds heme from the endogenous heme pool of E. coli [38]. Therefore, fusion of the PsbF protein to the LexA DNA binding domain (LexA DBD) allows one to measure the interaction of the PsbF transmembrane region within the membrane and, to study the influence of single amino acid substitutions on the transmembrane helix interaction propensity. The GALLEX system was designed to measure such homo- as well as hetero-oligomeric interactions of transmembrane helices within the E. coli cytoplasmic membrane [8].

Oligomerization of a transmembrane helix of interest results in formation of a LexA DBD dimer, which can bind to a promoter/operator region. This promoter/operator, which is integrated into the E. coli genome, controls the expression of a reporter gene (lacZ) and the propensity of a transmembrane domain to oligomerize can be measured by determining the activity of the reporter β-galactosidase. In the GALLEX assay the reporter gene activity is repressed if a transmembrane domain oligomerizes.

In Fig. 1A the oligomerization propensity of the wt PsbF peptide is determined using the GALLEX assay. As controls, the interaction of the wt glycoporphin A transmembrane helix (strong interaction) and the glycoporphin A transmembrane helix with a single amino acid substitution (G83I, very weak interaction) is measured. Besides measuring the interaction tendency of wt PsbF and PsbF H23A, the interaction of a PsbF variant G34I was also measured, since this amino acid substitution highly disturbs interaction of the wt glycophorin A transmembrane helix (strong interaction) and the glycophorin A transmembrane helix with a single amino acid substitution (G83I, very weak interaction) is measured. Besides measuring the interaction tendency of wt PsbF and PsbF H23A, the interaction of a PsbF variant G34I was also measured, since this amino acid substitution highly disturbs assembly of cytochrome b559 [38]. The presented results reproduce the findings of a recent study [38] and demonstrate that PsbF oligomerizes within the E. coli membrane, although to a lesser extend than the glycoporphin A transmembrane helix. Interestingly, after substitution of histidine 23, which is involved in heme binding, by alanine no significant differences in interaction propensity was observed (Fig. 1A). Since it has been shown in vitro that such a substitution of this residue abolishes heme binding completely [38], this observation indicates that heme binding does not affect dimerization of the PsbF peptide significantly.

While the GALLEX assay allows measuring the interaction of transmembrane helices in vivo, the heme redox state cannot be controlled and thus a potential influence of the heme redox state on cytochrome assembly cannot be measured. To study the effect of the heme redox state on association of the PsbF peptide in vitro, a PsbF-MalE fusion protein was used, which has been shown to form stable dimers on SDS gels [38]. Although the protein forms dimers under SDS-PAGE conditions, this approach does not allow studying the influence of heme binding or the heme redox state on dimerization since the non-covalently attached heme is released during electrophoresis. To overcome this problem the fusion protein was incubated in detergent in the absence of heme, or in the presence of oxidized or reduced heme prior to electrophoresis and a cross-linking reagent was added to stabilize the assembled dimers during SDS-PAGE. As a control, the protein was also incubated without heme and in the presence of 250 mM SDS, which prevents dimerization. As can be seen in Fig. 1B, a significant amount of the PsbF fusion protein forms homodimers in vitro but the presence of either reduced or oxidized heme does not appear to have any significant effect on dimerization of the fusion protein as can be deduced from the intensity of the monomer and dimer bands on Coomassie stained gels.

To elucidate any influence of the large MalE fusion domain on dimerization and since the SDS-PAGE approach provides only a rough estimate of the influence of heme binding on oligomerization, chemically synthesized peptides were used to further study dimerization in more detail by resonance energy transfer.

### 3.2. Synthesis and in vitro reconstitution

For the present study several ways to assemble cytochrome b559 from chemically synthesized PsbF peptides in detergent solutions were tested. A mixture of DDM and SDS at a mole fraction of around 0.4 (5 mM DDM and 4 mM SDS) resulted in best assembly of cytochrome b559. In Fig. 2 a spectrum of reduced and...
speed of 240 nm/min.

Elmer Lambda 25 spectrophotometer with a resolution of 0.5 nm and a scan reduced by addition of 5 mM sodium dithionite. Spectra were taken on a Perkin-

Formation of cytochrome b559 in 50 mM Tris pH 8.0, 50 mM NaCl, 5 mM DDM, 3.5 mM SDS was added to oxidized cytochrome b559 after in vitro reconstitution is shown. Formation of cytochrome b559, which displays defined absorbance maxima, suggests that the monomeric PsbF peptide can form a homodimer in vitro and binds the heme cofactor.

For measuring oligomerization of chemically synthesized PsbF peptides in vitro, different PsbF variants were produced, each labeled with a fluorescent dye, in order to monitor association by fluorescence resonance energy transfer (FRET).

3.3. Specific dimerization of PsbF in micelles

The influence of the site-selectively incorporated fluorophores, via a single cysteine residue at the C-terminus of the PsbF peptide, on dimerization of the PsbF transmembrane helix was studied by competition assays and analyzed by SDS-PAGE. Unlabeled and labeled peptides were mixed with the PsbF-MalE fusion protein that forms stable homodimers on SDS-gels (Fig. 3). Addition of unlabeled peptide results in loss of the dimer band (lane B) as does addition of the fluorescent labeled peptides (lanes C and D). This observation suggests that the PsbF helices are able to exchange with one another and that dye labeling does not significantly interfere with in vitro dimerization. Surprisingly, we did not observe a heterodimer between the PsbF-MalE fusion protein and the peptides on SDS-gels, which has a calculated molecular weight of 52.5 kDa. Most likely the heterodimer has approximately the same apparent molecular weight on an SDS gel as the PsbF-MalE protein alone.

In order to measure energy transfer between the two PsbF peptides within a homodimer, a fluorescent donor and acceptor pair was chosen whose absorbance does not overlap with the inherent absorbance of cytochrome b559. This is necessary to minimize possible interference from absorbance of the cytochrome moiety. BODIPY 577/618 was chosen as a donor fluorophore, since it has a high extinction coefficient and it has been shown in several studies that BODIPY fluorophores are photo-stable and relatively insensitive to solvent polarity and pH. In addition, BODIPY derivatives have already been used to study interaction of transmembrane helices in vitro [39]. It is worth noting that the BODIPY fluorophore spectrum can change when fluorophore monomers are in close proximity to each other [40,41]. Initially, we attempted to exploit this effect and use solely BODIPY labeled PsbF to follow oligomerization but we were not able to observe spectral changes in our steady state fluorescence measurements, as it has also been reported for the M13 major coat protein [39]. Therefore, a second peptide was synthesized that carries the fluorophore DY633 as an acceptor.

The excitation and emission spectra of the BODIPY and DY633 labeled peptides are shown in Fig. 4. Although the absorbance maximum of BODIPY in buffer is 580 nm, the donor still absorbs light at wavelengths > 590 nm with high efficiency. For all further experiments the donor was excited at 595 nm. At this wavelength there is essentially no absorbance from the cytochrome moiety anymore (compare Fig. 2). Nevertheless, the acceptor dye is also slightly excited at this wavelength. To test whether fluorescence quenching of BODIPY and/or fluorescence emission of DY633 after excitation at 595 nm can be used to measure energy transfer, the emission spectra of separately sensitized donor and acceptor peptides were measured, integrated, and compared to the emission spectrum obtained after mixing of the two populations at a ratio of 1:1 (Fig. 4B). Mixing of the two differently labeled peptides led to a decrease in donor fluorescence over a period of 1.5 h whereas the emission of the acceptor increased (Fig. 4B). Formation of the PsbF heterodimers requires dissociation of the two homodimer populations and association of the differently labeled peptides, which took about 1.5 h under the chosen experimental conditions. These results demonstrate that the two differently labeled peptides can undergo an exchange reaction with each other and that emission changes at 628 nm and/or 651 nm can be used to measure oligomerization of the PsbF peptide by fluorescence quenching through energy.

It has been shown previously that fluorescence quenching can be used to analyze the oligomeric structure of single helices in detergent or lipid vesicles [14,42,43]. The SDS gels shown in Figs. 1 and 3 demonstrate that PsbF forms homodimers in SDS.
The dependence of energy transfer between the two dyes on the mole fraction acceptor can be used to determine the number of peptides associated within an oligomer. In Fig. 5 fluorescence quenching at 628 nm is displayed as a function of acceptor mole ratio at a constant total peptide concentration and a constant peptide to detergent ratio. The data have been fitted with a least square method. The energy transfer depends linearly on the acceptor mole fraction and this clearly indicates that the oligomer formed is a dimer. As derived in [14], the amount of quenched donor is proportional to $1 - (P_{PsbF-BODIPY})^n$, where $P_{PsbF-BODIPY}$ is the mole ratio of BODIPY labeled peptide and $n$ is the oligomer number. For this calculation, equal energy transfer to all subunits was assumed. This assumption does not allow to determine the accurate oligomeric state of larger oligomers but does permit distinguishing between dimers and higher oligomers, since the dimer is the only case where quenching vs. mole fraction acceptor is always linear [14,43]. Therefore, it can be concluded that PsbF forms a dimer in micelles (Fig. 5). This conclusion is consistent with the observation that PsbF also forms dimers on SDS gels (compare Figs. 1 and 3) and no higher ordered oligomers were observed.

This fluorescence assay provides a valuable tool for testing the influence of cofactor binding on helix–helix interactions between PsbF monomers. Any changes in helix–helix interaction should result in increased or decreased FRET and thus can be e.g. measured as fluorescence quenching at 628 nm.

3.4. Heme binding, the heme redox state, and helix–helix interactions

In a recent study it was shown that formation of holo-cytochrome b$_{559}'$ is a three step process [38]: after integration of the polypeptide into a membrane (step I), the helices interact to form the homodimeric apo-cytochrome (step II) and subsequently the cofactor is bound (step III). Cytochrome b$_{559}'$ formation can therefore be described with the following scheme:

$$\text{membrane insertion} \rightarrow 2M \rightarrow [D] + H \rightarrow [DH]$$

For the formation of holo-cytochrome b$_{559}'$ two dissociation constants have to be considered ($K_{D1}$ and $K_{D2}$). So far we only measured energy transfer caused by dimerization of the monomeric PsbF peptides without cofactor, which is described by $K_{D1}$.

In principle, measuring the energy transfer between differentially labeled transmembrane helices at various peptide concentrations allows determination of the dissociation constant of transmembrane helices in vitro [42,44,45]. Thus, measuring FRET at various concentrations of labeled PsbF protein should allow determination of the PsbF dissociation constant $K_{D1}$ in detergent if the dissociation constant of the PsbF peptide is sufficiently low. However, due to the fact that the labeled PsbF peptide cannot be dissolved in buffer at concentrations >5 μM, which appeared to be below the $K_{D1}$, we were not able to obtain a sufficient complete titration curve and to subsequently determine $K_{D1}$. It can only be estimated that $K_{D1}$ is >5 μM (data not shown).

After addition of heme to the PsbF peptide a spectrum typical for cytochrome b$_{559}'$ can be measured, which proves that heme is incorporated into the homodimeric apo-cytochrome b$_{559}'$ ([D] in Scheme 1) and, therefore, after addition of heme the equilibrium is shifted to the holo-form ([DH] in Scheme 1). If addition of

![Fig. 4. (A) Fluorescence spectra of labeled PsbF peptides in detergent (PsbF-BODIPY and PsbF-DY633). Excitation (solid lines) and emission spectra (dotted lines) were taken at a peptide concentration of 2 μM with excitation $\lambda_{ex}=595$ nm and emission $\lambda_{em}=660$ nm. (B) Fluorescence emission was measured at $\lambda_{em}=595$ nm for the donor and acceptor peptide alone at a concentration of 2 μM peptide and the two individual spectra were integrated (dotted line). An equimolar mixture of donor and acceptor peptides at a total peptide concentration of 4 μM was incubated at room temperature for 2 h and fluorescence emission was measured subsequently (solid line).](image)

![Fig. 5. PsbF association in micelles. Fluorescence quenching at 628 nm is plotted as a function of the acceptor mole fraction. The total peptide concentration was kept constant at 4 μM, while the ratio of PsbF-BODIPY and PsbF-DY633 was varied.](image)
In the recent years, peptide based models have been used to extensively study in vitro assembly of designed b-type cytochromes and these studies have allowed to gain insights into structural requirements for the formation of b-type cytochromes (for recent reviews see [46,47]). However, always idealized proteins have been used, which were derived from a transmembrane b-type cytochrome and converted to a soluble protein. Although such an approach is of great value for studying assembly of idealized soluble b-type cytochromes, caution must be applied in transferring these findings on transmembrane b-type cytochromes since the forces, that drive folding and assembly of a-helical transmembrane proteins, are different. Besides using peptide based models, assembly of several soluble b-type cytochromes has been studied to some extend and the influence of heme binding and the heme redox state on assembly and stability of these proteins has been analyzed [48,49].

Here we present an analysis of the in vitro assembly of a transmembrane b-type cytochrome and analyze the influence of heme binding and the heme redox state on assembly of a minimal transmembrane b-type cytochrome. The PsbF homodimer displays a rather simple architecture with only two helices forming a transmembrane helix dimer and the protein can serve as an excellent model system to study assembly of a simple transmembrane b-type cytochrome. Based on a recent analysis, it has been suggested that assembly of holo-cytochrome b559 within the membrane is a two step process (Scheme 1). In the first step the PsbF monomers interact to form an apo-cytochrome and in the second step the heme cofactor binds [38]. In contrast to soluble proteins, formation of a hydrophobic core is not a driving force for membrane protein folding, since the membrane (or micellar) environment is hydrophobic. Also no special hydrophobic pocket has to form to accommodate the cofactors and there is no need to highly rearrange the structure for heme incorporation or binding, at least in the case of the apo-cytochrome b559 homo-dimer. But does heme binding promote or disturb dimerization of the PsbF peptides? For cytochrome b559 we did not observe an influence of the heme binding on the dimerization propensity of the PsbF protein. By using a genetic approach, we could show that the wt PsbF peptide dimerizes within a membrane (Fig. 1A and [38]). In addition, after substitution of the axial heme ligand histidine 36 by alanine, essentially the same tendency to interact was measured in the membrane when compared to the wt PsbF protein [38]. This observation is in good agreement with the further result that addition of heme does not cause a strong increase of BODIPY quenching and, therefore, does not result in a dramatic increase of PsbF dimer formation.

It is possible that the equilibrium described by $K_{D2}$ does not only depend on the concentration of the homodimeric apo-cytochrome [D] and free heme [H] but also on the redox state of the heme cofactor. PsbF dimer stabilization or destabilization, depending on the heme redox state, could result in increased or decreased donor fluorescence quenching. As can be seen in Fig. 6, we did not observe any significant influence of the heme redox state on fluorescence quenching. Incorporation of either oxidized or reduced heme increases helix dimerization to the same extent. Therefore we conclude that neither the heme redox state nor heme binding drives cytochrome b559 formation in vitro, but cytochrome formation is mainly driven by the interaction of the transmembrane helices.

### 4. Discussion

So far not much is known about the in vivo assembly of b-type cytochromes and even less about the influence of transmembrane helix–helix interactions and cofactor binding on transmembrane b-type cytochrome assembly.
peptide abolishes formation of potential higher ordered structures and because of this only dimers were observed (Fig. 5). If this is true, the pathway of cytochrome $b_{559}$ formation could differ in vivo. But it is reassuring that the results from the genetic approach and the SDS-PAGE approach (Fig. 1) as well as the FRET data are conclusive and indicate the same pathway of cytochrome $b_{559}$ formation.

Assembly of the holo-cytochrome is mainly driven by transmembrane helix interactions and heme binding does not notably shift the individual assembly steps (Scheme 1) towards a dimeric state. In contrast to cytochromes with a more complex structure, in the case of cytochrome $b_{559}$ the heme is not deeply buried within a protein core but is still accessible to detergent or membrane lipids. This could lead to an entirely different effect of the heme incorporation on the assembly of this transmembrane b-type cytochrome, when compared to formation and stability of larger transmembrane helix bundles. Furthermore, partitioning of the heme group out of the more hydrophilic environment into the hydrophobic protein core is most likely also not a driving force for the assembly of cytochrome $b_{559}$ and, more in general, of transmembrane b-type cytochromes.

A thermodynamic study on the energetics of heme binding to the soluble cytochrome $b_{562}$ has shown that heme binding significantly contributes to the stability of the holo-protein [50]. This observation indicates that the heme redox state can influence formation of soluble b-type cytochromes significantly. In contrast, the data obtained with the SDS-PAGE assay (Fig. 1B) as well as the FRET study (Fig. 6) suggest that the heme redox state does not affect dimerization of the PsbF peptide. The monomer–dimer equilibrium is not highly affected by the formation of holo-cytochrome $b_{559}$ and heme binding does not promote dimerization of PsbF. Therefore, helix–helix interactions mainly drive assembly of cytochrome $b_{559}$.

As a conclusion, the observations of this study suggest important differences between assembly of soluble and transmembrane b-type cytochromes. Nevertheless, since cytochrome $b_{559}$ displays a rather simple architecture when compared to other b-type cytochromes, there should be extreme caution in extrapolating this study’s findings in the folding of other transmembrane b-type cytochromes with a more complex structure.

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